United States Department of Agriculture
Agricultural Marketing Service, Science & Technology
Microbiological Data Program

SOP No.: MDP-LABOP-07		Page 1 of 1
Title: Maintenance of *Salmonella* and *E.coli* Positive Control Cultures with GFP Plasmid		
Revision: Original	Replaces: N/A	Effective: 09/01/03

1. Purpose:

To provide standard procedures for maintenance of Green Fluorescent Plasmid-bearing (GFP) positive control strains for USDA/AMS Microbiological Data Program (MDP).

2. Scope:

This standard operating procedure (SOP) shall be followed by all laboratories conducting microbiological studies for MDP, including support laboratories conducting non-routine activities that may impact the program.

Positive control strains bearing the Green Fluorescent Plasmid (GFP) will be inoculated into a plastic bag containing produce and eluate and cultured in parallel with regular MDP analytical samples. Successful recovery of the control strains (as opposed to naturally-contaminating isolates) from the analytical procedures can be determined by visualization of GFP by irradiation of the cultures with ultraviolet light at 365 nM.

3. Outline of Procedure:

5.1 Equipment and Materials

5.2 Media and Reagents

5.3 Description of Strains

5.4 Maintenance of Strains

5.5 Positive in-run Control Cultures

5.6. Characteristics of *Escherichia coli* O137:H41 Strain MW421 (USDA/ARS/PW #RM2375) (transformed #RM3658) from cabbage root

5.7. Characteristics of *Salmonella enterica* serovar Poona CDHS/MDL #00A 3563 (USDA/ARS/PW #RM235)

4. References:

4.1 Miller, W.G, J.H. Leveau and S.E. Lindow. 2000. Improved gfp and inaZ broad-host-range promoter-probe vectors. Mol. Plant-Microbe Interact. 13: 1243-1250.

4.2 Barak, J.D., L.C. Whitehand, and A.O. Charkowski. 2002. Differences in attachment of *Salmonella enterica* serovars and *Escherichia coli* O157:H7 to alfalfa sprouts. Appl. Environ. Microbiol. 68: 4758-4763.

4.3 Andrews, W.H., Sherrod, P.S., Hammack, T.T., and Amaguana, R.R., 1998. Food and Drug Administration Analytical Manual (BAM), 8th ed. (revision A). George J. Jackson (Ed). AOAC International, Gaithersburg, MD 20877 pp. App 3.64

4.4 SOP MDP-LABOP-02, Sample Wash Procedures

4.5 SOP MDP-LABOP-07 Attachment 1, Heart Infusion Agar with 40 ug/mL Kanamycin (example form)

4.6 SOP MDP-LABOP-02 Attachment 2, Kanamycin Stock Solution @ 500 mg/mL (example form)

5. Specific Procedures:

5.1 Equipment and Materials

5.1.2 Postive bacterial strains bearing GFP Plasmid pKT-kan as described in section 5.3 below.

5.1.3 Materials, supplies, media and samples required for testing routine MDP produce samples.

5.2 Media and Reagents

5.2.1 Nutrient agar slants with kanamycin sulfate (40 mg/liter)

5.2.2 Nutrient agar Petri plates with kanamycin sulfate (40 mg/liter)

5.2.3 Stock kanamycin solution: 1mL of Sterile Kanamycin Stock Solution (200mg/ml)

- 200mg Kanamycin sulfate (Fisher Catalog # BP906-5 (5 grams) or equivalent in 1mL distilled water
- Mix to dissolve

- Filter solution through a 0.2µm syringe filter (syringe -B-D # 309602 filter-Fisher Catalog # 09-719C or equivalent), into a sterile screw cap tube.

  NOTE: Due to absorption of fluid by filter material the final volume is <1mL;

5.2.3.1 Sterile solution should be stored at 4°C or frozen (-80°C), for long term storage

5.2.3.2 0.2mL per liter is added to sterile culture media for a 40µg/mL final concentration.

5.2.4 Preparation of Media with Antibiotic

5.2.4.1 Broth Media (Nutrient broth, Brain Heart Infusion broth, etc…)

5.2.4.1.1 Make media as per manufacturer instructions

5.2.4.1.2 Do not dispense into test tubes prior to autoclaving/boiling and aseptic addition of kanamycin stock solution.

5.2.4.1.3 After autoclaving/boiling as instructed by manufacturer cool media to ~50°C

5.2.4.1.4 Aseptically add Kanamycin Stock Solution at 0.2ml per liter, mix gently (40µg/ml final concentration).

5.2.4.1.5 Dispense into aseptically sterile test tubes

5.2.4.2 Agar Media (Nutrient agar, Brain Heart Infusion agar, etc …)

5.2.4.2.1 Make media as per manufacturer instructions.

5.2.4.2.2 After autoclaving/boiling as instructed by manufacturer cool media to ~50°C

5.2.4.2.3 Aseptically add Kanamycin Stock Solution, 0.2ml per liter (40µg/ml final concentration)

5.2.4.2.4 Dispense as desired into sterile test tubes or petri plates

5.2.5 All media required for testing routine MDP produce samples including extra media needed for QC culture as listed below.

Lactose broth	3- 450 ml bottle 1- 100 ml bottle
Tetrathionate/IKI broth	3 tubes
Selenite-cystine broth	3 tubes
M-broth	6 tubes
VIDAS	3 tests
Lauryl Tryptose broth, double-strength (DS LTB)	5 tubes
Lauryl Tryptose broth, single-strength (SS LTB)	5 tubes
EC Broth	10 tubes

5.3 Description of Control Strains

5.3.1 Plasmid pKT-kan [W. Miller (ref. 1)] is a stable, broad-host range vector that confers kanamycin resistance and green fluorescent protein (GFP) expression. GFP is visualized under UV light at 365 nM.

5.3.2 Plasmid pKT-kan in which a 131-bp *npt11* promoter fragment from Tn5 was fused to the green fluorescent protein gene (gfp) of plasmid pPROBE-KT, is a stable, broad-host-range vector that confers kanamycin resistance and green fluorescent protein expression. (ref. 1).

5.3.3 Plasmid pKT-kan was transformed into the following enteric bacterial strains:

5.3.3.1 *E. coli* O137:H41 Strain MW 421 (USDA/ARS/PW #RM2375, ref 2.) (transformed #RM3658), a cabbage root isolate (working designation: *E.coli* #3658 pKT-kan.)

5.3.3.2 CDHS/MDL #00A 3563 (USDA/ARS/PW #RM2350) *Salmonella enterica* serovar Poona, (working designation: S.Poo #2350 pKT-kan.) a human clinical isolate from an outbreak associated with cantaloupe (ref. 2.)

5.4 Maintenance of Strains

5.4.1 Plasmid pKT-kan transformed enteric strains for positive-culture controls will be received by MDP laboratories grown-up on slants of Nutrient agar with kanamycin (40 mg/liter).

5.4.2 Strains should be grown on a nutrient-type agar containing kanamycin to maintain selective pressure favoring the transformed biotype. Bacteria with the plasmid pKT-Kan can be passed a few times without selective pressure of the antibiotic kanamycin. But to ensure the stability of the plasmid is maintained, it is best to maintain the antibiotic selective pressure on the organism.

5.4.3 Strains may be maintained or stored according to individual laboratory procedures for maintaining stocks of reference or control enteric cultures.

5.5 Significance of GFP Fluorescent observations in various media.

The fluorescence from the bacteria with the pKT-kan is green but the intensity can vary. S. Poona pKT-kan can have a greater intensity than the *Escherichia coli* pKT-kan. The intensity frequently is enhanced after holding the cultures at room temperature for 1-2 hours following routine incubation.

The fluorescence of GFP may be detectable in broth cultures but it depends on bacteria concentration, intensity of UV light used, and degree of darkness in the room. Some green fluorescence is usually detectable in most broth cultures.

The GFP fluorescence can be quenched by some media components. Quenching of fluorescence is most often observed where bacterial growth results in colony colorization or a precipitation to be formed on differential media.

Escherichia coli pKT-kan	Quenching of Flourescence
• EMB	Quenched
• MacConkey Agar Plates	Quenched
• XLD	No quenching
• Nutrient Agar	No quenching
• LIA	Quenched
• TSI	No quenching ▪ Slant fluoresces
• SIM	No quenching

Salmonella Poona pKT-kan	
• EMB	No quenching
• MacConkey Agar Plates	No quenching
• XLD	Partially quenched ▪ Colony edges fluoresce
• Nutrient Agar	No quenching
• LIA	Quenched
• TSI	Partially quenched ▪ Slant fluoresces ▪ H_2S quenches fluorescence
• SIM	No quenching

5.6. Characteristics of *Escherichia coli* O137:H41 Strain MW421 (USDA/ARS/PW #RM2375) (transformed #RM3658) from cabbage root

API 20E Identification Code # 5 044 572

BBL Enterotube II Identification Code # 34560

Biochemical Reactions

Glucose	+
Gas	+
Lysine	+
Ornithine	-
H_2S	-
Adonitol	-
Lactose	+
Arabinose	+
Sorbitol	+
Dulcitol	-
Phenylalanine	-
Urea	-
Citrate	-
ONPG	+
Arginine	-
Trytophane	-
Indole	+

VP	-
Gelatin	-
Mannitol	+
Inositol	-
Rhamnose	+
Sucrose	+
Melibiose	+
Amygdalin	-
Oxidase	-
Motility	+

5.7. Characteristics of *Salmonella enterica* serovar Poona CDHS/MDL #00A 3563 (USDA/ARS/PW #RM235)

API 20E Identification Code # 6 704 552

BBL Enterotube II Identification Code # 37070 or 37071

Biochemical Reactions

Glucose	+
Gas	+
Lysine	+
Ornithine	+
H_2S	+
Adonitol	-
Lactose	-

Arabinose	+
Sorbitol	+
Dulcitol	+
Phenylalanine	-
Urea	-
Citrate	+
ONPG	-
Arginine	+
Trytophane	-
Indole	-
VP	-
Gelatin	-
Mannitol	+
Inositol	-
Rhamnose	+
Sucrose	-
Melibiose	+
Amygdalin	-
Oxidase	-
Motility	+

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ORIGINAL SIGNATURE PAGE MAINTAINED BY USDA, AMS, SCIENCE & TECHNOLOGY, MONITORING PROGRAMS OFFICE
ELECTRONICALLY REPRODUCED SIGNATURES

- Established procedures for maintenance of Green Fluorescent Plasmid-bearing (GFP) positive control strains

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Revision: Original	Replaces: N/A	Effective: 09/01/03

Purpose- For maintenance of bacterial plasmids.

Order-

Date ordered:	Date needed:	Amount needed:	Initials:

Standard order amount- 1 liter (Makes 32 plates or 200 tubes)

Recipe -

Ingredients:	For 1 liter:	Amount used:	Mfr:	Lot #:	Exp date:
HI broth powder	25 grams				
Agar	20 grams				
Kanamycin monosulfate stock solution (500 mg/mL)	80 ul				
DI water	1 liter				

Preparation-

1. Suspend HI broth powder and agar in 1 liter of DI water. Mix thoroughly.
2. Boil for one minute to dissolve completely.
3. Measure pH. Adjust if necessary with 0.5 N HCl or 1.0 N NaOH.
4. Autoclave 15 min. @ 121°C and 15 PSI. Cool agar to 50°C in a waterbath for 20 min.
5. Add 80 ul sterile kanamycin suspension. Mix well.
6. Dispense 30 ml per standard petri plate. Leave lids slightly ajar until plates cool.
   Or dispense 5 ml into screw cap tubes and cool in a slanted position.

7. Agar should be light to medium amber.
8. Label and date.

Required pH:	Original pH:	Final pH:
7.4 ± 0.2 @ 25°C		

Volume of acid or base added, if needed, to obtain final pH: _____
Lot # of acid- _____ Exp. date- _____ Lot # of base- _____ Exp. date- _____

Prepared by- Int- _____ Date prepared- _____ Amount prepared- _____

Storage- Temperature-2-8°C; Where- Ref. # 1; Shelf life-6 months; Exp. date-_____
(In screw cap tubes or plates in a plastic bag)

Quality Control-

Sterility- Pass Fail (circle one) Date completed- _____ Int. ____

Performance- Pass Fail (circle one) Date completed- _____ Int. ____

Comments- _____
_____

Purpose- Ingredient for HI agar with kanamycin.

Order-

Date ordered:	Date needed:	Amount needed:	Initials:

Standard order amount- 1 liter (Makes 32 plates or 200 tubes)

Recipe -

Ingredients:	For 1 liter:	Amount used:	Mfr:	Lot #:	Exp date:
Kanamycin monosulfate	1.0 gram				
DI water					

Preparation-

9. Dissolve 1.0 g kanamycin monosulfate in 2 ml DI water (1000mg/2mL = 500 mg/mL).
10. Pipet into a sterile syringe that has a sterile 0.2 um filter on the luer-lok tip.

11. Dispense into a sterile 2 ml microcentrifuge tube that has a screw cap.

Prepared by- Int- _____ Date prepared- _____ Amount prepared- _____

Storage- For short term storage, keep at 4 °C.
For long term storage, keep at –20 °C.

Quality Control- None required.

Comments- _____
_____